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Additional Vinyl Ketones and Their Pyranyl Ketones in Gonyleptid Harvestmen (Arachnida: Opiliones) Suggest These Metabolites Are Widespread in This Family

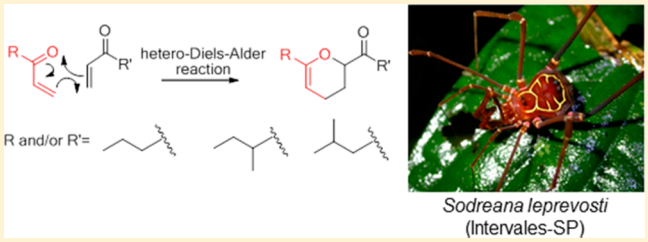
Felipe C. Wouters,[†] Daniele F. O. Rocha,[†] Caroline C. S. Gonçalves,[†] Glauco Machado,[‡] and Anita J. Marsaioli*[,†]

[†]Chemistry Institute, State University of Campinas, Campinas, SP, Brazil

[‡]Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil

Supporting Information

ABSTRACT: Four species of gonyleptid harvestmen, *Acanthogonyleptes pulcher*, *Gonyleptes saprophilus* (Gonyleptinae), *Sodreana barbiellini*, and *Sodreana leprevosti* (Sodreaninae), were examined by GC-MS and ^{1}H NMR. All of these species release vinyl ketones, and three of them produce the corresponding pyranyl ketones, which are presumed hetero-Diels−Alder (HDA) dimers. The vinyl ketones 5-methyl-1-hexen-3-one, *rac*-4-methyl-1-hexen-3-one, and (*S*)-4-methyl-1-hexen-3-one were synthesized. Natural 4-methyl-1-hexen-3-one is present as a single stereoisomer and has the *R*-configuration. Vinyl ketone dimers (HDA dimers) were also observed in the scent gland exudate and characterized by HRMS, ^{13}C NMR, and ^{1}H NMR chemical shifts of the pyranyl moiety.



Opiliones, also known as harvestmen, are arachnids with lateral exocrine glands located on the cephalothorax that release volatile compounds[1] that are known to shield harvestmen against natural predators, such as ants, frogs, lizards, and spiders.[2,3] In the suborder Laniatores, the composition of scent gland exudates can contain benzoquinones, alkyl phenols, and/or vinyl ketones.[4−6] We have recently reported the dimer of the α,β-unsaturated ketone 1-hepten-3-one, 1-(6-butyl-3,4-dihydro-2*H*-pyran-2-yl)pentanone (**1**), which was detected in the defensive secretions of two harvestman species (*Iporangaia pustulosa* and *Neosadocus maximus*) and is proposed to be a hetero-Diels−Alder (HDA) dimer.[4] Such dimerization generally requires specific conditions (i.e., high pressure and temperature) not present in harvestman scent glands, thus suggesting an enzymatically controlled HDA reaction in harvestmen.[4] Dimeric natural products containing pyranyl rings that are present in plant species, such as *Arnica sachalinensis* (Asteraceae)[7] and *Isodon rubescens* var. *rubescens*,[8] are also proposed to be HDA dimers.

Here we describe the chemical characterization of the exudates of four harvestman species belonging to the family Gonyleptidae. The chemical composition of the exudate was recently used as characteristic to construct the phylogeny of this large family.[6] The examined species produce a variety of vinyl ketones. The vinyl ketones 5-methyl-1-hexen-3-one (**2**) and 4-methyl-1-hexen-3-one (**3**) were synthesized and fully characterized by GC-MS and NMR spectroscopy. We also report spectroscopic evidence of the presence of four minor vinyl ketones (1-hexen-3-one (**4**), 4-methylhexan-3-one (**5**), 4,5-dimethylheptan-3-one (**6**), and 4-methyl-1-hexen-3-one (**7**)) and seven different HDA dimers in the scent gland exudates of three of the four harvestmen (Table 1).

RESULTS AND DISCUSSION

MS was the major tool to discover 5-methyl-1-hexen-3-one (**2**) in *Sodreana barbiellini* (Mello-Leitão) and *Sodreana leprevosti* (B. Soares & H. Soares) and 4-methyl-1-hexen-3-one (**3**) in *Gonyleptes saprophilus* (Mello-Leitão) and *Acanthogonyleptes pulcher* (Mello-Leitão) defensive secretions (Table 1, Figure 1). The MS spectrum of vinyl ketone **2** showed a molecular ion at *m*/*z* 112 and fragments at *m*/*z* 55 and 70 arising from a ketone α-cleavage and a McLafferty rearrangement, respectively. Likewise, vinyl ketone **3** possessed a molecular ion at *m*/*z* 112 but was distinguished by a fragment at *m*/*z* 84 rationalized as a McLafferty rearrangement. NMR characterization of **3** was not available in the literature; however, the *G. saprophilus* exudate was clean enough to make a full NMR spectroscopic analysis (Table 2). The ^{1}H NMR spectrum showed signals at 5.77, 6.27, and 6.44 ppm assigned to hydrogens on C-1 and C-2 with characteristic couplings of a terminal double bond (Table 2). The two diastereotopic hydrogens at C-5 appear as two ensembles of seven peaks interpreted as partially overlapped double doublets of quartets. The relative and absolute configuration of this ketone was assessed by first establishing a protocol to discriminate the enantiomers of a racemic synthetic sample. The same synthetic pathway was established for **2** and **3**. The 3-methylbutanoic acid and 2-methylbutanoic

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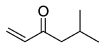
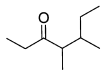
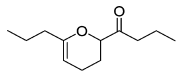
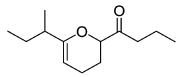
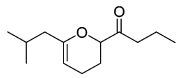
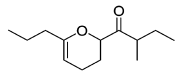
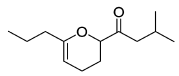
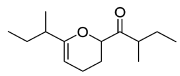
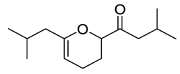
Table 1. Compounds Detected in Defensive Secretion of Gonyleptid Harvestmen

Compound	RI	characteristic ions (*m/z*, abundance)	Species	Rel. abundance
 1-hexen-3-one (**4**)	742	98(M^+,13), 70(14), 55(100), 43(16), 41(14)	*Gonyleptes saprophilus* *Sodreana barbiellini* *Sodreana leprevosti*	5.2% 3.4% 2.0%
 5-methyl-1-hexen-3-one (**2**)	828	112(M^+,11), 97(24), 70(71), 57(16), 55(100), 43(10), 41(20)	*Sodreana leprevosti* *Sodreana barbiellini*	69.1% 31.4%
 4-methyl-1-hexen-3-one (**3**)	831	112(M^+,15), 97(12), 84(35), 83(12), 69(12), 58(28), 56(23), 55(100), 41(29)	*Acanthogonyleptes pulcher* *Gonyleptes saprophilus* *Sodreana barbiellini*	100.0% 69.0% 0.1%
 4-methylhexan-3-one (**5**)	835	114(M^+,14), 85(10), 57(100), 41(14)	*Gonyleptes saprophilus* *Sodreana barbiellini*	0.1% 0.1%
 4,5-dimethylheptan-3-one (**6**)	856 [a]	86(6), 58(42), 57(100), 55(11), 43(10), 41(24)	*Gonyleptes saprophilus*	0.1%
 4-methyl-1-hepten-3-one (**7**)	922	126(M^+,1), 84(97), 83(31), 71(14), 70(11), 56(16), 55(100), 43(58), 41(19)	*Gonyleptes saprophilus*	0.2%
 1-(6-(propyl)3,4-dihydro-2*H*-pyran-2-yl)butanone (**8**)	1299	HREIMS (M^+)196.1479 (calcd for $C_{12}H_{20}O_2$, 196.1463)[b] 196(M^+,48), 125(100), 107(27), 97(15), 83(19), 81(22), 79(22), 69(15), 55(75), 43(49), 41(24)	*Sodreana barbiellini*	0.7%
 1-(6-(1-methyl-propyl)3,4-dihydro-2*H*-pyran-2-yl)butanone (**9**)	1376	210(M^+,76), 139(100), 125(90), 121(30), 95(90), 93(35), 83(33), 81(28), 69(60), 57(39), 55(76), 43(68), 41(45)	*Gonyleptes saprophilus*	1.6%
 1-(6-(isopropyl)3,4-dihydro-2*H*-pyran-2-yl)butanone (**10**)	1378	HREIMS (M^+) 210.1630 (calcd for $C_{13}H_{22}O_2$ 210.1620)[b] 210(M^+,62), 139(100), 125(65), 97(18), 95(53), 93(33), 83(22), 79(25), 69(29), 67(17), 55(44), 43(49), 41(31)	*Sodreana barbiellini* *Sodreana leprevosti*	9.5% 0.5%
 1-(6-propyl-3,4-dihydro-2*H*-pyran-2-yl)2-methyl-butanone (**11**)	1386	HREIMS (M^+) 210.1630 (calcd for $C_{13}H_{22}O_2$ 210.1620)[b] 210(M^+,65), 139(62), 125(100), 107(30), 83(24), 81(30), 79(26), 69(23), 57(40), 55(92), 43(40), 41(40)	*Gonyleptes saprophilus*	1.5%
 1-(6-propyl-3,4-dihydro-2*H*-pyran-2-yl)isobutanone (**12**)	1388	HREIMS (M^+) 210.1633 (calcd for $C_{13}H_{22}O_2$ 210.1620)[b] 210(M^+,64), 139(83), 125(100), 107(31), 97(23), 83(29), 81(27), 79(27), 69(20), 57(25), 55(88), 43(42), 41(31)	*Sodreana barbiellini* *Sodreana leprevosti*	8.1% 0.5%
 1-(6-(1-methyl-propyl)3,4-dihydro-2*H*-pyran-2-yl)2-methyl-butanone (**13**)	1462	HREIMS (M^+) 224.1783 (calcd for $C_{14}H_{24}O_2$ 224.1776)[b] 224(M^+,41), 140(11), 139(100), 121(17), 95(22), 93(17), 83(13), 81(11), 69(24), 57(36), 55(30), 43(12), 41(21)	*Gonyleptes saprophilus*	21.7%
 1-(6-isopropyl-3,4-dihydro-2*H*-pyran-2-yl)isobutanone (**14**)	1479	HREIMS (M^+) 224.1811 (calcd for $C_{14}H_{24}O_2$224.1776)[b] 224(M^+,31), 140(10), 139(100), 97(10), 95(25), 93(16), 85(11), 83(11), 79(11), 69(12), 57(16), 55(18), 41(16),	*Sodreana barbiellini* *Sodreana leprevosti*	46.7% 27.9%

[a]Retention index obtained from linear regression from the other compounds. [b]HREIMS obtained by CG-TOF-MS.

acid acyl chlorides were transformed into *N*-methoxy-*N*-3-dimethylbutanamide derivatives, which produced **2** and **3**, respectively, by reaction with vinyl magnesium chloride. The same synthetic pathway was applied to obtain (*S*)-**3** using (*S*)-2-methylbutanoic acid as starting material. Thus (*S*)-**3** and (*rac*)-**3** were analyzed by GC-FID using a chiral phase column, and their co-injections revealed the retention times of (*S*)-**3** and (*R*)-**3**, 11.6 and 12.0 min, respectively. Co-injection of *G.*

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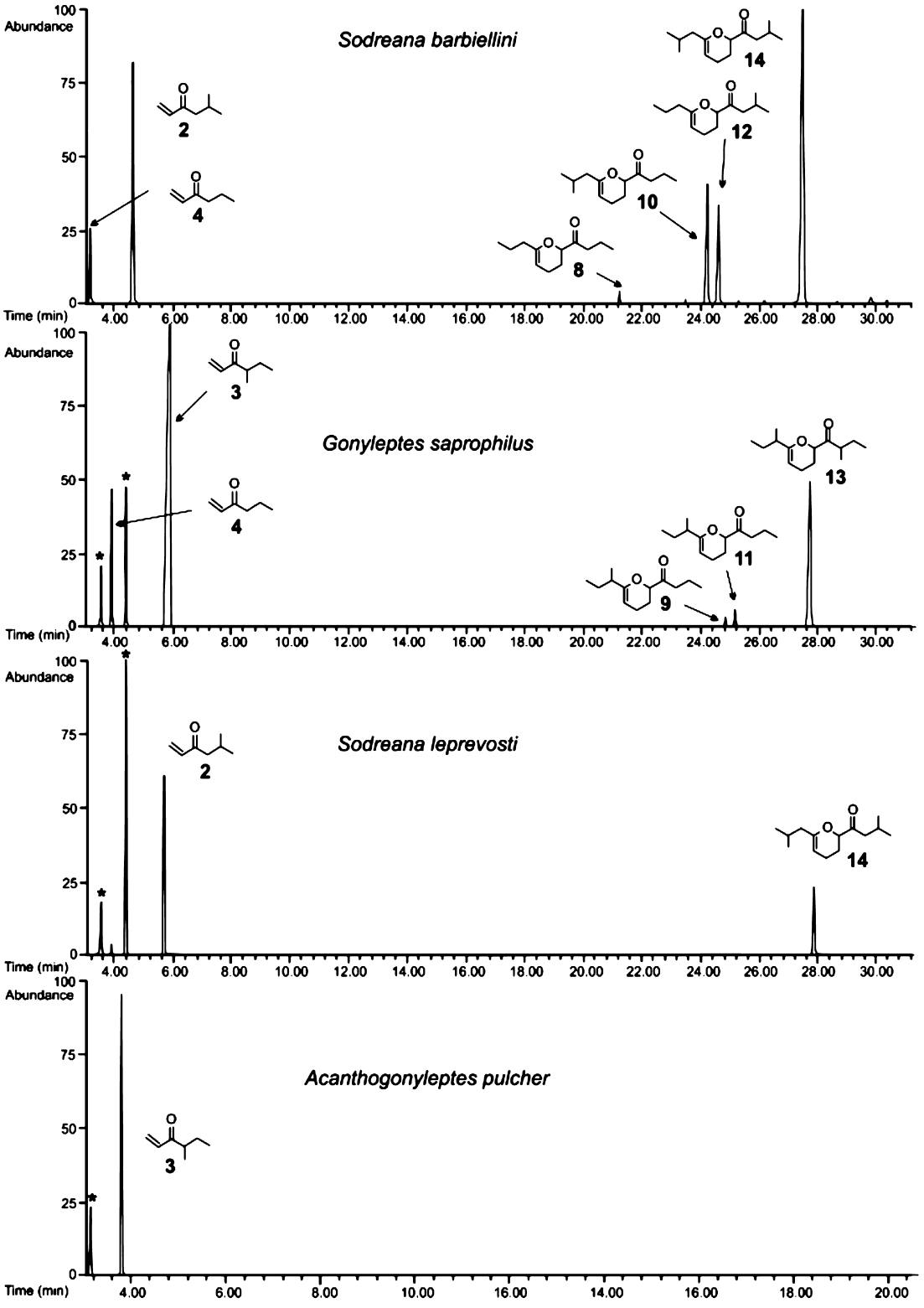

Figure 1. GC-MS analyses of gonyleptid harvestmen exudate containing vinyl ketones and their hetero-Diels–Alder dimers. Peaks labeled with * refer to solvent impurities. *A. pulcher* and *G. saprophitus* exudates were analyzed under two different conditions, but vinyl ketone **3** was confirmed by co-injection with a standard compound and MS.

saprophilus and *A. pulcher* exudates with the racemic standard showed that (*R*)-**3** is the enantiomer present in >99% enantiomeric excess (Figure 2). The absolute *S*-configuration of analogous 4-methyl-3-ketones from arthropods was previously determined for the (*S*)-4-methylheptan-3-one from the ant *Atta texana*[9] and (*S*)-4-methyl-1-hepten-3-one from the walking stick *Agathemera elegans*.[10]

HDA dimers **8**–**14** detected in *G. saprophilus*, *S. leprevosti*, and *S. barbiellini* (Figure 1, Table 1) were characterized by HREIMS and MS spectra according to the molecular ions and the fragmentation patterns of α-carbonyl cleavage followed by

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Table 2. NMR Spectroscopic Data ($CDCl_3$) for Natural 4-Methyl-1-hexen-3-one (3) from *Gonyleptes saprophilus*.

position	δ_C, type	δ_H (J in Hz)
1	128.1, CH_2	6.27, dd (17.5, 1.4)
		5.77, dd (10.5, 1.4)
2	135.5, CH	6.44, dd (17.5, 10.5)
3	204.4	
4	45.2, CH	2.42, qdd (7.5, 7.1, 7.1)
5	26.2, CH_2	1.72, ddq,[a] (14.0, 7.1, 6.9)
		1.42, ddq,[a] (14.0, 7.1, 6.9)
6	11.8, CH_3	0.89, t (7.5)
7	16.1, CH_3	1.10, d (6.9)

[a]These signals are partially superimposed double doublets of quartets that look like septets.

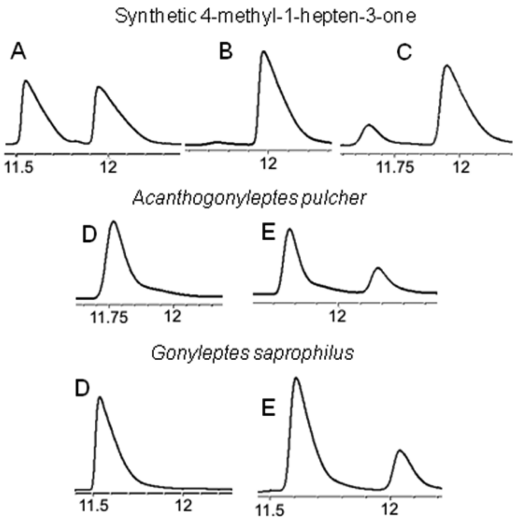

Figure 2. GC-FID analysis of 4-methyl-1-hexen-3-one (3). (A) racemic, (B) *S*-enantiomer, (C) *S*- and *rac*-, (D) natural sample, (E) natural and racemic sample.

CO loss (Figure 3). Their structures were proposed considering the presence of vinyl ketones in the same exudate and all alternatives of the HDA reactions between diene and dienophile. Compounds **9** and **11** present in *G. saprophilus* were suggested as heterodimers of **3** and **4** and were distinguished by their base peak at m/z 139 and 125, respectively, and the presence of the monomeric vinyl ketones. The structures of the heterodimers **10** and **12** found in *S. barbiellini* and *S. leprevosti* were suggested taking into consideration the presence of the vinyl ketones **2** and **4** and their base peak fragments at m/z 139 and 125, respectively. The presence of the pyranyl moiety in the homodimer **14** from the *S. barbiellini* exudate was confirmed by comparing the NMR data (^{13}C NMR and ^1H NMR) of the exudate with those of **1** (Table 3). Additionally, several signals of methyl groups at 23.5−22.9 ppm are consistent with a mixture of compounds with 3-methylbutan-1-one and isobutyl side chains.

Hara et al.[5] previously reported the same composition in the *S. leprevosti* (=*Zortalia inscripta*) exudate, but dimer **14** was described as unknown. The ketone **2** was detected in *Neosadocus maximus*, while **3** was present in *Gonyleptes curvicornis* and *Parampheres* sp. defensive secretions.[5] The tenebrionid *Amargmus tristis* also produces **2** as a component of its defensive secretion together with other defensive compounds.[11]

The presence of possible HDA dimers in several harvestman exudates raises the issue of a hetero-Diels−Alderase. The only confirmed Diels−Alderase has been isolated from the bacterium *Saccharopolyspora spinosa*,[12] and there is no report of an isolated and characterized hetero-Diels−Alderase. However, the presence of this enzyme in harvestmen and/or their symbiont microorganisms[13] seems to be responsible for the production of pyranyl ketones from the vinyl ketones, as this reaction typically requires forcing conditions to occur chemically. Additionally, the occurrence of the dimers depends on the presence of the vinyl ketones, while the inverse is not necessarily true, as observed for *A. pulcher* (Figure 1). The racemic nature of **1** however is not readily explained by an enzymatic reaction, and we have no other evidence than the fact that dimer formation is not spontaneous.[4]

The chemical profiles of *A. pulcher*, *G. saprophilus*, *S. barbiellini*, and *S. leprevosti* defensive secretions are consistent with those of other species of the suborder Laniatores,[6] showing vinyl ketones as major components (Table 1). Vinyl ketones were detected in representatives of the family Gonyleptidae, mainly in the subfamilies Gonyleptinae, Hernandariinae, Sodreaninae, Progonyleptoidellinae, and Caelopyginae.[4,5]

This work confirms the structure and absolute configuration of (*R*)-**3** in *G. saprophilus* and reports the presence of pyranyl ketones in three additional species of harvestmen (*S. barbiellini*, *G. saprophilus*, and *S. leprovosti*). Such data suggest that representatives of the family Gonyleptidae possess hetero-Diels−Alderases responsible for the biosynthesis of these secondary metabolites. Further molecular biology studies are necessary to prove the existence of this novel enzyme in harvestmen. The chemical profile of the defensive secretions and the relationships between the components give information about the chemical defense evolution and possible biosynthetic routes of harvestman secondary metabolites.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were acquired with either an 11 T Varian Inova instrument, operating at 499.88 MHz for ^1H NMR and 125.71 MHz for ^{13}C NMR, or a 5.87 T Bruker Avance DPX, at 250.13 MHz for ^1H NMR and 62.89 MHz for ^{13}C NMR. $CDCl_3$ was used as the solvent, and tetramethylsilane (TMS) as the internal reference (0.0 ppm). GC-MS analyses were performed using an Agilent 6890-5973 system using a DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm). EIMS spectra were recorded at 70 eV at a scanning speed of 3.54 scans s^{-1} from m/z 40 to 400. The oven temperature ranged from 50 to 200 °C at 10 °C min^{-1} and then to 290 °C at 16 °C min^{-1}. Natural samples (1 μL) were injected in the splitless mode and synthetic in split (1:10) mode. The injector temperature was 250 °C and the detector was 280 °C with helium as the carrier gas. The retention index (RI)[14] was determined using splitless mode and two different temperature programs: (A) *G. saprophilus*, *S. barbellini*, and *S. leprevosti* had the highly volatile 1-hexen-3-one (**4**), and the oven temperature ranged from 40 to 290 °C at a rate of 4 °C min^{-1} and 7.07 psi; (B) for the other analyzed species, the temperature ranged from 50 to 290 °C, at a rate of 4 °C min^{-1} and 7.62 psi. Separately, an alkane standard solution, C_8−C_{20} (Fluka), was injected in the same program (for 1-hexen-3-one (**4**) addition of heptane was needed). Enantioselective analyses were conducted with a GC-FID Agilent 6890 using stationary phase Chirasil-dex (25 m × 0.25 mm × 0.25 μm), H_2 at 0.5 mL min^{-1}, injector and detector at 220 and 250 °C, respectively. A volume of 1 μL of the samples was injected in the splitless mode. The temperature ranged from 40 to 100 °C at a rate of 3 °C min^{-1}, from 100 to 180 °C at a rate of 30 °C min^{-1}, and held at 180 °C for 20 min. HREIMS was performed on a Waters GCT Premier at 20 scans s^{-1}, resolution 7000 fwhm, sub-5 ppm RMS with

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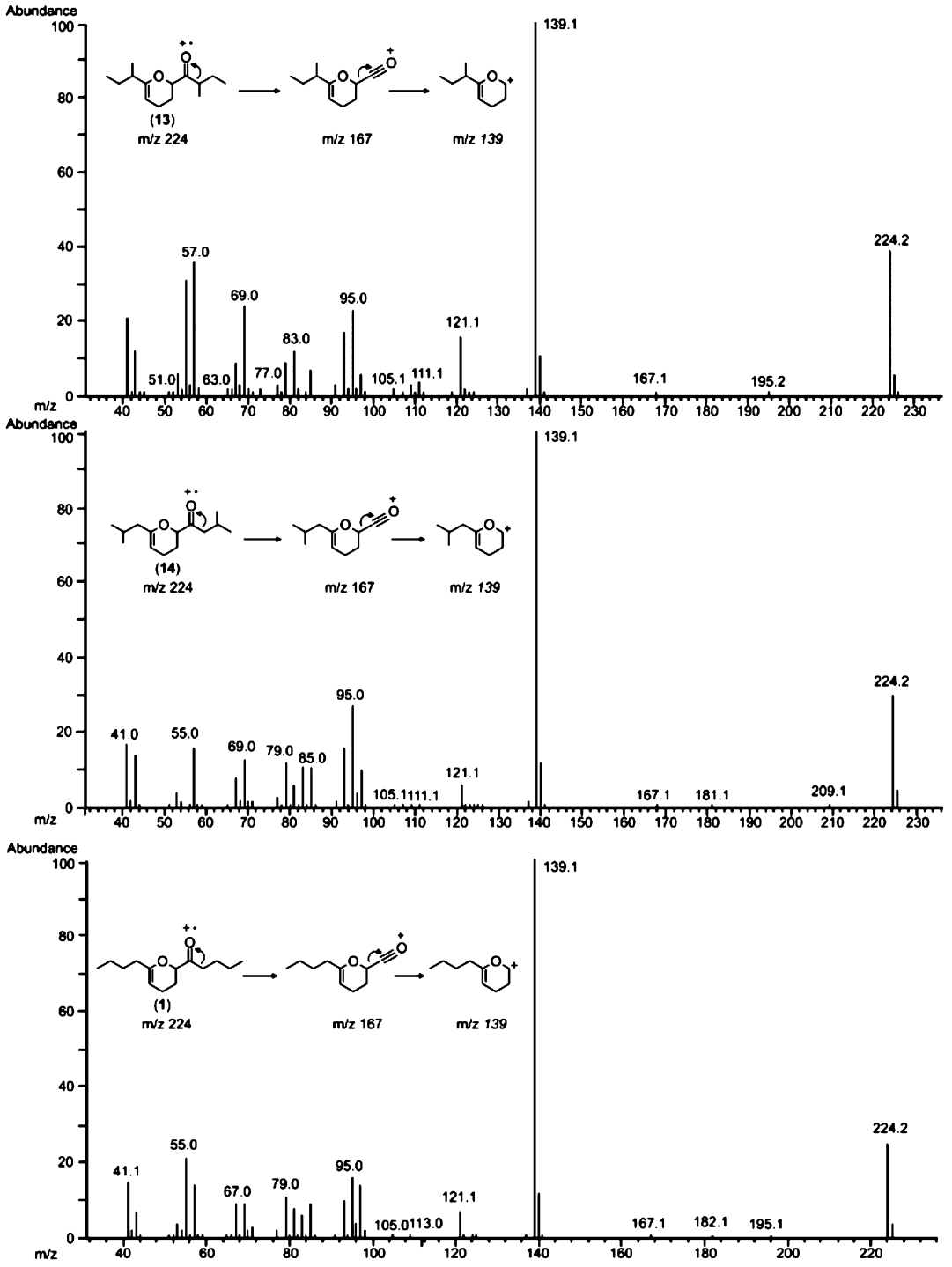

Figure 3. MS spectrum and fragmentation of HDA dimers **1**, **13**, and **14**.

internal lock mass correction and electron impact (EI) of 70 eV. The GC Agilent 7683 operated with an oven temperature ranging from 50 to 250 °C at 10 °C min^{-1} and an HP5-MS column (30 m × 0.25 mm × 0.25 μm). The injection volume was 1 μL in splitless mode. The injector temperature was 270 °C and the detector was 250 °C, using helium as the carrier gas.

Secretion Sampling. Individuals of each species were collected in different places of the Atlantic Forest in southeastern Brazil. *A. pulcher* was collected in the Estação Biológica do Alto da Serra (23°46′ S; 46°20′ W), municipality of São Paulo, in January 2010. *G. saprophilus* was collected in the vicinity of the Parque Nacional do Itatiaia (22°20′ S; 44°35′ W), along the border of the states of Minas Gerais and Rio de Janeiro, in November 2010. *S. barbiellini* was collected in the Fazenda Capricórnio (23°22′ S; 45°04′ W), municipality of São Paulo, in February 2011. Finally, *S. leprevosti* was collected in the Parque Estadual Intervales (24°14′ S; 48°04′ W), municipality of Ribeirão Grande, state of São Paulo, in November−December 2009. Voucher specimens of all studied species are deposited in the Museu de Zoologia (MZSP), Universidade de São Paulo, Brazil.

Individuals of all studied species were taken to the laboratory and kept alive in plastic vials containing a piece of wet cotton to maintain moisture. The defensive secretions were collected by pressing the gland openings with cotton wool previously cleaned with doubly distilled EtOAc. Each individual produced 1 to 5 μL of defensive secretion mixed with enteric fluid. The liquid absorbed in the cotton wool was washed with $CDCl_3$ (2 mL) for NMR analyses and eluted

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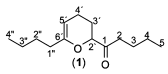
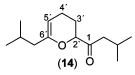
Table 3. Characteristic ^1H and ^{13}C NMR Signals for HDA Adduct 14 in *Sodreana barbiellini* Exudate (C_6D_6) and 1-(6-Butyl-3,4-dihydro-2*H*-pyran-2-yl)pentan-1-one (1, $CDCl_3$, ref 5)



(1)



(14)

Position[a]	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
2′	80.2, CH	4.25, dd (3.1, 8.6)	80.9, CH	4.02, dd (3.0, 8.5)
5′	95.6, CH	4.52, t (3.4)	97.7, CH	4.40, t (4.0)
6′	153.5, C	-	153.2, C	-

[a]Results from ^1H and ^{13}C (fully decoupled DEPT-135) and 2D HSQC (^1H–^{13}C, ^1J) spectra of the concentrated *Sodreana barbiellini* exudate.

with EtOAc (2 mL) for GC-MS analyses. All solvents were of high analytical grade and doubly distilled before use. The number of individuals of each species was as follows: *A. pulcher* (n = 5), *G. saprophilus* (n = 15), *S. barbiellini* (n = 5), *S. leprevosti* (n = 11).

Synthesis. *N-Methoxy-N-3-dimethylbutanamide (**15**).* 3-Methylbutanoic acid (9.06 mmol, 1 mL) was mixed with dimethylformamide (35 μL) and cooled in an ice bath. Thionyl chloride (10.42 mmol, 0.76 mL) was added dropwise, and the system was allowed to reach room temperature (rt) and mixed for 1 h. The excess of HCl was removed by following nitrogen flux, and $CHCl_3$ (75 mL) and *N,O*-dimethyl hydroxylamine (9.97 mmol, 0.972 g) were added. The system was cooled, and pyridine (22.65 mmol, 2.2 mL) was added. After 1 h at rt, the reaction mixture was washed with H_2O and dried with $MgSO_4$, and the pyridine was removed as an azeotropic mixture with heptane. The product was purified by column chromatography with silica gel and a gradient of hexane/EtOAc as mobile phase, giving 0.68 g of a colorless oil, in 52% yield. ^1H NMR (250.13 MHz, $CDCl_3$) δ 3.68 (3H, s), 3.18 (3H, s), 2.30 (2H, m), 2.09–2.25 (1H, m), 0.97 (6H, d, ^3J = 7.5 Hz); ^{13}C NMR (62.89 MHz, $CDCl_3$) δ 174.1 (C, CO), 61.2 (CH_3,OCH_3), 40.7 (CH_2), 25.1 (CH), 22.7 (CH_3); EI-MS m/z 145 [M^+] (13), 85(77), 61(40), 58(11), 57(100), 43(12), 41 (35); GC retention time (min) 6.57.

*N-Methoxy-N,2-dimethylbutanamide (**16**).* Following the procedure described above and using 2-methylbutanoic acid (9.06 mmol, 1 mL), 1 g of the title compound was obtained as a colorless oil in 76% yield. ^1H NMR (250.13 MHz, $CDCl_3$) δ 3.69 (3H, s), 3.19 (3H, s), 2.75–2.84 (1H, m), 1.58–1.81 (1H, m), 1.39–1.47 (1H, m), 1.11 (3H, d, ^3J = 6.9 Hz), 0.89 (3H, t, ^3J = 7.4 Hz); ^{13}C NMR (62.89 MHz, $CDCl_3$) δ 171.3 (C, CO), 61.6 (CH_3, OCH_3), 37.0 (CH), 32.5 (CH_3, NCH_3), 27.0 (CH_2), 17.3 (CH_3, $HCCH_3$), 12.2 (CH_3, H_2CCH_3); EI-MS m/z 145(9) [M^+], 85(45), 61(16), 57(100), 41(22); GC retention time (min) 6.37.

*5-Methyl-1-hexen-3-one (**2**).* A solution of vinyl bromide (approximately 3 mL) in anhydrous tetrahydrofuran (THF, 10 mL) was added to a suspension of Mg^0 (39.5 mmol, 0.96 g) in THF (10 mL). The reaction was heated to reflux, and after total consumption of the magnesium, the solution was transferred to a vessel containing **15** (2.07 mmol, 300 mg) and 3 mL of THF. The reaction was stirred for 16 h at rt and quenched with saturated NH_4Cl. After extraction with ethyl ether, the organic phase was dried with anhydrous $MgSO_4$ and the solvent removed by fractional distillation. The final product was a solution of the title compound in THF. Ketone **2** was first described in ref 15.

*4-Methyl-1-hexen-3-one (**3**).* The procedure was similar to that described above, substituting **16** for **15**. The pure *S*-enantiomer was obtained from (*S*)-**16** using silylated glassware with freshly distilled TMS-Cl. The product was obtained as a THF solution. Retention times (Chirasil-dex column): (*S*)-**16**, 11.55 min; (*R*)-**16**, 12.0 min. The ^1H NMR spectrum had THF signals, and **3** did not endure distillation; therefore we have used the solution in GC-MS analyses.

■ ASSOCIATED CONTENT

Supporting Information

Spectroscopic characterization of all natural and synthetic compounds is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +55-19-35213067. Fax: +55-19-35213023. E-mail: anita@iqm.unicamp.br.

Notes

The authors declare no competing financial interest.

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